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Polyphenolic composition of extracts from winery by-products and effects on cellular cytotoxicity and mitochondrial functions in HepG2 cells

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ABSTRACT

Winery by-products arise in high amounts during winemaking processes. Hence, recovery alternatives are of great interest. In this study, effects of extracts from winery by-products (*Vitis vinifera* L. cv. Riesling) on mitochondrial functions in human hepatocellular carcinoma (HepG2) cells were examined. Polyphenolic profiles of pomace (PE), stem (SE), vine leaf (VLE), and vine shoot extracts (VSE) were characterized by HPLC-UV/Vis-ESI-MS/MS. The extracts induced dose-dependent cytotoxic effects (PE > SE > VLE > VSE). VSE showed protective effects regarding modulation of *tert*-butyl hydroperoxide (TBH)-induced intracellular reactive oxygen species (ROS) levels. PE, SE and VLE increased the mitochondrial membrane potential (MMP), whereas VSE decreased it owing to mildly impaired mitochondrial respiration. Cells may try to compensate reduced respiration chain complex activities by increasing the mitochondrial mass, as indicated by enhanced citrate synthase activity and mRNA expression levels after VSE incubation. Thus, winery by-products represent interesting sources of bioactive compounds that exert positive or negative effects on mitochondrial functions.

1. Introduction

The estimated world production of wine in 2017 was more than 24 billion liters (Organisation Internationale de la Vigne et du Vin). Of these, approximately 1 billion liters were produced in Germany, with predominantly white grape varieties being cultivated, of which *Vitis vinifera* L. cv. Riesling was the most dominant (Statistisches Bundesamt, 2019a, 2019b). During the winemaking process, winery by-products are formed, such as pomace, stems, leaves and shoots (Makris, Boskou, & Andrikopoulos, 2007). For example, vine shoots occur in huge amounts at pruning in springtime and are traditionally used as a natural fertilizer (Luque-Rodriguez, Perez-Juan, & Luque de Castro, 2006). Owing to their content of secondary plant metabolites, such as polyphenols, usage of winery by-products as sources of bioactive compounds offers an opportunity to obtain value-added products for the food, cosmetics and pharmaceutical industry (Martins et al., 2011). Polyphenols in winery by-products include hydroxybenzoic acids, hydroxycinnamic acids and their respective derivatives, stilbenes and flavonoids, such as flavanols, flavonols, dihydroflavonols and anthocyanins, the latter mainly occurring in winery by-products of red grape varieties (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Antoniolli, Fontana, Piccoli, & Bottini, 2015; Lambert et al., 2013; Püssa, Floren, Kuldkepp, & Raal, 2006). It is well known that polyphenols protect cells against oxidative stress due to antioxidative and radical scavenging abilities and are associated with numerous beneficial health effects. A broad spectrum of biological activities of polyphenol-rich winery by-product extracts has been investigated, including antioxidant, antiproliferative and anti-inflammatory activities (Hogan, Canning, Sun, Sun, & Zhou, 2010; Jara-Palacios et al., 2015; Rodriguez-Morgado et al., 2015). As cellular reactive oxygen species (ROS) production is closely related to mitochondrial processes, the effects of polyphenols on mitochondrial functions are of particular interest. Mitochondria are highly dynamic organelles involved in various cellular processes, such as energy metabolism, redox regulation and cell death pathways (Brown & Borutaite, 2012; Duchen, 2000; Frank et al., 2001; Susin, Zamzami, & Kroemer, 1998). Production of adenosine triphosphate (ATP) through oxidative phosphorylation is one of the main functions of mitochondria (Midzak, Chen, Aon, Papadopoulos, & Zirkin, 2011). Since ROS are mainly generated by electrons escaping from complexes I and III of the electron transport chain, leading to formation

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of superoxide radical anion (O_2^-) (Selivanov et al., 2011), mitochondria are the main cellular source of ROS but also a target of them (Simon, Haj-Yehia, & Levi-Schaffer, 2000). ROS can cause mitochondrial damage, associated with a reduced mitochondrial membrane potential (MMP), followed by intracellular ATP depletion (Wang, Green, & Simpkins, 2001). Some effects of polyphenols on mitochondrial processes have already been investigated, such as the ability to inhibit complexes of the mitochondrial respiratory chain (Hodnick, Bohmont, Capps, & Pardini, 1987; Lagoa, Graziani, Lopez-Sanchez, Garcia-Martinez, & Gutierrez-Merino, 2011; Zini, Morin, Bertelli, Bertelli, & Tillement, 1999) and influence mitochondrial biogenesis via the Sirtuin 1 (SIRT1)-PGC-1α pathway (Lagouge et al., 2006).

The aim of the present study was to prepare extracts from winery by-products (pomace, stem, leaves and vine shoots) of the white grape variety *Vitis vinifera* L. cv. Riesling, provided by a conventional viticulturist in Rhineland Palatinate, Germany, and to study their chemical composition and biological activities *in vitro* in HepG2 cells, focusing on mitochondrial functions. Although, not used to represent hepatocytes, HepG2 cells were employed as an *in vitro* model to investigate cytotoxicity in general (Giampieri et al., 2018). Furthermore, due to high contents of organelles, HepG2 cells represent an established model for investigating mitochondrial parameters (Esselun, Bruns, Hagl, Grewal, & Eckert, 2019).

2. Materials and methods

2.1. Chemicals, cells and media

Chemicals and solvents were of analytical grade or compliant with standards required for cell culture experiments. Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). HepG2 cells were obtained from DSMZ (Braunschweig, Germany). Hydrochloric acid (HCl) was purchased from CHEMSOLUTE®, Th. Geyer GmbH & Co. KG (Renningen, Germany), methanol and acetonitrile Optigrade HPLC gradient grade were from LGC Standards (Wesel, Germany), trypsin and triton X-100 were from Serva (Heidelberg, Germany), and triethanolamine from VWR (Radnor, PA, USA). ε-Viniferin, *trans*-polydatin, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-glucoside, *trans*-caftaric acid and *trans*-coutaric acid were purchased from Phytolab (Vestenbergsgreuth, Germany). Acetyl-CoA was purchased from AppliChem (Darmstadt, Germany). Black 96-well plates (flat clear bottom) from Corning® were also purchased via Sigma Aldrich (Taufkirchen, Germany). An ATPlite luminescence assay kit and CulturPlate-96 microplates (white) were purchased from PerkinElmer (Waltham, MA, USA) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA), eriodictyol and digitonin were from Fluka (Deisenhofen, Germany). Gallic acid was purchased from Alfa Aesar (Haverhill, MA, USA) and naringenin from Carl Roth (Karlsruhe, Germany). RPMI 1640 cell culture medium and supplements (fetal calf serum (FCS), penicillin/streptomycin) were purchased from Life Technologies GmbH/ThermoFisher Scientific (Darmstadt, Germany). Cell culture materials (clear well plates, flasks, etc.) were from Greiner Bio-One (Essen, Germany). RNA Protect, an RNeasy mini kit and RNase-free water were purchased from Qiagen (Hilden, Germany). Primers were from Biomol (Hamburg, Germany), an iScript cDNA synthesis kit from BioRad (Munich, Germany) and TURBO DNA-free™ kit from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Plant material

Winery by-products of *Vitis vinifera* L. cv. Riesling were provided by a conventional viticulturist in Rhineland-Palatinate (Weingut Schenk-Siebert, Grünstadt-Sausenheim, Germany) and originated from a single vineyard (Sausenheimer Höllenpfad). Grapevine leaves, stems and pomace were provided in 2014 and stored at −20 °C until extraction. Vine shoots were cut from the same vineyard during pruning in springtime 2016 and stored dry at room temperature until extraction.

2.3. Preparation of extracts

The preparation of extracts was performed as reported previously (Fuchs et al., 2019). Lyophilized and grinded winery by-products were extracted twice in a ratio of 1 g of solid per 25 ml of extraction solvent (pomace, stems, grapevine leaves: methanol 100%; vine shoots: methanol/water/1N HCl: 80/19/1; v/v/v) at room temperature under stirring (1st: 60 min, 2nd: 30 min). After sterile filtration (0.2 µm), the extraction solvent was removed under reduced pressure at 40 °C by rotavapor and the residue was transferred to a water phase. To remove sugars, salts and organic acids, solid-phase extraction using Amberlite® XAD16N resin (Sigma Aldrich, Taufkirchen, Germany) was carried out. After conditioning with ethanol (90%) and equilibration with double distilled water, the column was loaded with the aqueous extract and washed with 5 bed volumes of double distilled water. Retained substances were eluted with 5 bed volumes of ethanol (90%). The eluate was evaporated under reduced pressure at 40 °C, transferred to a water phase and lyophilized. The powdery extracts were homogenized and stored in the dark at −20 °C until use.

2.4. Total phenolic content (TPC)

The TPC of extracts was determined by the spectrophotometric method of Folin-Ciocalteu (Bobo-García et al., 2015) with slight modifications. As gallic acid was used as a phenolic standard, a six-point gallic acid standard calibration curve was prepared (10 µg/ml − 200 µg/ml, dissolved in dimethyl sulfoxide (DMSO)). In brief, 10 µl of diluted extract (100–200 µg/ml, dissolved in DMSO) or gallic acid solution and 100 µl of 10% Folin-Ciocalteu reagent were added to a 96-well microplate. After 5 min incubation at room temperature, 80 µl sodium carbonate (Na_2CO_3, 100 g/l) was added. Absorbance was measured at 750 nm by a microplate reader after 2 h incubation in the dark at room temperature. The TPC was expressed as micrograms of gallic acid equivalent per gram of dry extract (mg GAE/g extract).

2.5. Characterization of polyphenolic profiles

Characterization of the polyphenolic profiles of the extracts was performed by HPLC-DAD and HPLC-UV/Vis-ESI-MS/MS. Via HPLC-UV/Vis-ESI-MS/MS identifications of substances were performed by specific fragmentation patterns, whereby HPLC-DAD analysis furthermore enabled comparisons of UV/Vis-spectra of compounds contained in the extracts with these of available reference substances as well as literature data. The HPLC-DAD analysis was performed using an Agilent 1200 series HPLC system. Polyphenols were separated using a Synergi 4 µm polar-RP column (80 Å, 250 × 4.6 mm, Phenomenex, Torrance, USA) at 30 °C with 0.1% aqueous formic acid (A) and acetonitrile (B) as solvents and a flow rate of 0.8 ml/min. The gradient profile started at 10% B, was kept isocratic for 9 min, increased to 25% B within 1 min and then kept isocratic for 9 min. Over the next 15 min, B was raised to 50% and then after 6 min, B was raised to 98%, after which the column was rinsed for 10 min. Subsequently, the column was rinsed with the initial solvent conditions (10% B) for 10 min. The extracts were measured at a concentration of 1 mg/ml in methanol/formic acid (99.9/0.1; v/v) with 10 µl injection volume. UV/Vis-detection was performed from 200 nm to 650 nm. Simultaneous monitoring was conducted at 280 nm (hydroxybenzoic acids, flavanols), at 320 nm (hydroxycinnamic acids, stilbenes) and at 360 nm (flavonols). Identification of the polyphenolic compounds in the extracts was made using an Agilent 1100 series HPLC-UV/Vis (Agilent, Santa Clara, California, USA) coupled to API 2000 triple quad mass spectrometer (Sciex, Framingham, MA, USA). Precursor ion and product ion scans were recorded. The HPLC conditions were the same as in the HPLC-DAD analysis. The extracts were measured at concentrations of 1 mg/ml and 2 mg/ml in methanol/

formic acid (99.9/0.1; v/v) with an injection volume of 20 µl. UV-detection was performed at 320 nm. The ESI-MS/MS conditions in the negative ion mode were as follows: nebulizer gas: 60 psi; heater gas: 45 psi; curtain gas: 35 psi; collision gas: 6 psi; ion spray voltage: −3000 V; temperature: 400 °C; declustering potential: −35 to −120 V; focusing potential: −250 V; entrance potential: −4.5 V to −11 V; collision cell entrance potential: −9.4 V to −20 V.

2.6. Cell culture and incubation of HepG2 cells with extracts

Human hepatocellular carcinoma HepG2 cells were cultivated in 175 cm^2 flasks in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin and were grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded in culture plates and after a 24 h growing period were incubated with extracts (1–500 µg/ml dissolved in DMSO, final solvent concentration ≤ 1.0%) in FCS reduced medium (5% FCS) for 24 h incubation or FCS-free medium for 2 h incubation. To exclude effects of the solvent DMSO on the test systems media controls were carried along. To avoid artifactual generation of extracellular hydrogen peroxide (H_2O_2) by pro-oxidative interaction of phenolic compounds with media constituents (Lapidot, Walker, & Kanner, 2002), incubations of HepG2 cells were performed in the presence of catalase (100 U/ml). To exclude deviations in the test systems caused by cytotoxicity, further investigations were performed using extract concentration ranges corresponding to cell viability > 80% according to the alamarBlue assay.

2.7. Cytotoxicity (alamarBlue assay)

Cytotoxicity was determined by the alamarBlue assay, which measures metabolic activity from the reduction of resazurin to resorufin (fluorescent). Thus, cell viability was determined according to mitochondrial integrity and cell growth (O'Brien, Wilson, Orton, & Pognan, 2000). Briefly, cells were seeded in clear 48-well plates (24 h: 1.8×10^5 cells/well; 2 h: 2.0×10^5 cells/well) and cultivated for 24 h. After incubation with extract (5–500 µg/ml dissolved in DMSO, final concentration 1.0%), cells were washed with phosphate buffered saline (PBS) and treated for 1 h with serum-free medium (500 µl) containing 10% resazurin solution. Fluorescence was measured by a Synergy 2 microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany; ex 530 nm, em 590 nm, 37 °C). Sodium dodecyl sulfate (SDS; 0.1%) was used as a positive control. Results were expressed as relative cell viability as a percentage of that for the solvent control.

2.8. Intracellular adenosine triphosphate (ATP) level

An assay based on firefly (*Photinus pyralis*) luciferase (ATPLite luminescence assay system, Perkin Elmer, Waltham, MA, USA), which catalyzes the formation of light from ATP and luciferin, was used to determine effects on intracellular ATP levels. As the emitted light in this assay is linearly related to the ATP concentration, intracellular ATP levels can be assessed by measuring luminescence (Crouch, Kozlowski, Slater, & Fletcher, 1993). Briefly, cells were seeded in white 96-well plates (24 h: 2.0×10^4 cells/well; 2 h: 2.5×10^4 cells/well) and cultivated for 24 h. After incubation with extract (1–500 µg/ml dissolved in DMSO, final concentration ≤ 1.0%), cells were washed with PBS, then 100 µl cell culture medium and 50 µl lysis buffer were added to each well and the plate was shaken at room temperature for 5 min (700 rpm). Subsequently, 50 µl of substrate solution containing luciferase and luciferin was added under reduced light and the plate was shaken at room temperature for another 5 min (700 rpm). After 10 min of dark adaptation, luminescence was measured using a Synergy 2 microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 10 µM) was used as a positive control. Results were expressed as the relative intracellular ATP level as a percentage of that for the solvent control.

2.9. Intracellular ROS levels (dichlorofluorescein (DCF) assay)

Modulation of *tert*-butyl hydroperoxide (TBH)-induced intracellular ROS levels after extract incubation was assessed by the DCF assay (Wang & Joseph, 1999) with slight modifications (Schaefer et al., 2006). Briefly, cells were seeded in black 96-well plates (clear flat bottom; 24 h: 2.0×10^4 cells/well; 2 h: 2.5×10^4 cells/well) and cultivated for 24 h. After incubation with extract (1–200 µg/ml dissolved in DMSO, final concentration 1.0%), cells were washed with PBS and incubated for 30 min with 50 µM 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) (dissolved in DMSO: 0.5% v/v in PBS, pH 7.0). Afterwards, cells were washed and incubated with TBH (250 µM in PBS pH 7.4) for 40 min. The increase of fluorescence (FI) resulting from the oxidation of non-fluorescent 2′,7′-dichlorodihydrofluorescin (DCFH) to fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular ROS was measured at 0 and 40 min using a Synergy 2 microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany; ex 485 nm, em 528 nm, 37 °C). Dye and oxidant incubations, washing steps and fluorometric determination were performed under the exclusion of light. FI was calculated as [((F40 min − F0 min)/F0 min) * 100] and results were expressed as the relative FI as a percentage of that for the TBH control.

2.10. Measurement of mitochondrial membrane potential (MMP)

MMP was measured using tetramethylrhodamine ethyl ester (TMRE), a cationic fluorescent dye that accumulates in mitochondria due to the negatively charged mitochondrial matrix. By measuring cellularly retained dye, average MMP can be estimated (Bernardi, Scorrano, Colonna, Petronilli, & Di Lisa, 1999). Briefly, cells were seeded in black 96-well plates (clear flat bottom; 24 h: 1.0×10^5 cells/well; 2 h: 1.5×10^5 cells/well) and cultivated for 24 h. After incubation with extract (1–200 µg/ml dissolved in DMSO, final concentration 1.0%), cells were washed with PBS and incubated with 500 nM TMRE (dissolved in DMSO: 0.5% v/v in FCS-free cell culture media) in the dark for 30 min. Afterwards, cells were washed with PBS and fluorescence was measured using a Synergy 2 microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany; ex 530 nm, em 590 nm, 37 °C). FCCP (20 µM) was used as a positive control. Results were expressed as relative MMP as a percentage of that for the solvent control.

2.11. High-resolution respirometry (HRR) analysis

Mitochondrial respiration was analyzed as described previously but with slight modifications (Hagl et al., 2015). Cells were incubated with VSE (250 µg/ml dissolved in DMSO, final concentration 0.5%) in 75 cm^2 cell culture flasks for 24 h. Afterwards, cells were harvested by trypsination and resuspended in MiR05 medium (cell density: 1×10^6 cells/ml). MiR05 is a mitochondrial respiratory medium developed by Oroboros Instruments (Innsbruck, Austria) (Stadlmann et al., 2006) consisting of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 0.5 mM), magnesium dichloride (3 mM), lactobionic acid (60 mM), taurine (20 mM), potassium dihydrogenphosphate (10 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 20 mM), sucrose (110 mM), and essential fatty acid free bovine serum albumin (1 g/l). Mitochondrial respiration was investigated using the Oxygraph-2 k system and DatLab software version 7.3.0.3 (Oroboros Instruments, Innsbruck, Austria). Functions of the respiratory system were examined by using a complex protocol, including the addition of different substrates, inhibitors and uncouplers. After adding 2 ml of cell suspension, treated with either VSE or solvent (DMSO, 0.5%), the two chambers of the Oxygraph-2k were closed to stabilize respiration (endogenous). By adding digitonin (1 µg/10^6 cells), permeabilization of cellular plasma membrane was induced, whereas the mitochondrial

outer and inner membranes remained intact. The oxidative phosphorylation capacity was determined using complex I related substrates (CI; NADH: ubiquinone oxidoreductase) glutamate (G; 10 mM), malate (M; 2 mM), and adenosine diphosphate (ADP; 2 mM). After the addition of glutamate/malate, leak respiration (labeled leak (G/M)) corresponded to state 4 respiration, whereas after the addition of ADP, state 3 respiration (CI OXPHOS) was induced. OXPHOS (CI + CII) was determined by the addition of succinate (10 mM). By injecting FCCP in 0.5 µl steps to the chambers, uncoupling (ETS) of the mitochondrial respiration was achieved. Complex II (succinate dehydrogenase) respiration in a non-coupled state (CII_{ETS}) was examined using rotenone (0.5 µM). The oxygen consumption by enzymes outside the electron transport chain, called residual oxygen consumption (ROX), was determined by inhibiting complex III (coenzyme Q: cytochrome *c* – oxidoreductase) using antimycin A (2.5 µM). This parameter was subtracted from all respiratory parameters. Oligomycin (2 µg/ml) was added to determine state 2 respiration leak (oligomycin). Afterwards, tetramethyl-phenylenediamine (TMPD; 0.5 mM) and ascorbate (2 mM) were added to measure cytochrome *c* oxidase (COX) activity (CIV) and the autoxidation rate was examined using sodium azide (≥100 mM) to correct COX respiration (Gnaiger, 2001).

2.12. Citrate synthase (CS) activity

CS activity was determined as described previously (Hagl et al., 2015). Aliquots of cell suspensions in MiR05 medium, which were also used for HRR analysis, were immediately frozen and stored at −80 °C until analysis. A reaction medium containing 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.5% Triton X-100, 0.5 mM oxaloacetate, 50 µM ethylenediaminetetraacetic acid (EDTA), 0.31 mM acetyl coenzyme A, 5 mM triethanolamine hydrochloride and 0.1 M Tris-HCl was prepared and preheated alongside 200 µl of cell suspension at 30 °C for 5 min. Afterwards, the reaction medium and cell suspension were mixed and CS activity was measured for 200 sec at 412 nm using a Jasco V650 spectrophotometer (Jasco, Pfungstadt, Germany). Measurements were performed in duplicate. CS activity was calculated as nmol/(min*10^6 cells).

2.13. Quantitative real-time PCR (qRT-PCR)

Briefly, cells were seeded in 6-well plates (5.0×10^5 cells/well) and cultivated for 24 h. After incubation with extract (250 µg/ml VSE; dissolved in DMSO, final concentration 0.5%), cells were washed with PBS, harvested and centrifuged (5 min, 265 x *g*, 4 °C). Next, cells were stabilized in RNA Protect (Qiagen, Hilden, Germany), immediately frozen and stored at −80 °C until analysis. Total RNA was isolated using a RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 and 280 nm using a NanodropTM 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed from the ratio of absorbance 260/280 nm and 260/230 nm. Samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions to remove residual genomic DNA (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 1 µg RNA using an iScript cDNA synthesis kit (BioRad, Munich, Germany) and was stored at −80 °C. qRT-PCR was conducted using a CfX 96 Connect™ system (BioRad, Munich Germany). Oligonucleotide primer sequences, primer concentrations and product sizes are listed in Table 1. All primers were purchased from Biomol (Hamburg, Germany). cDNA for qRT-PCR was diluted 1:10 with RNase-free water (Qiagen, Hilden, Germany). All samples were analyzed in triplicate. PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 29 s. Gene expression was analyzed using the −(2ΔΔCq) method with BioRad CfX manager software and values were normalized to expression levels of GAPDH, beta-actin and PGK1.

Table 1
Oligonucleotide primer sequences, product sizes and primer concentrations for quantitative real-time PCR; all primers were manufactured by Biomol (Hamburg, Germany) bp: base pairs, conc.: concentration.

Primer	Sequence	Products size (bp)	Primer conc. (µM)
ACTB	5′-ggacttcgagcaagagatgg-3′ 5′-agcactgtgttggcgtacag-3′	234	200
ATP5D	5′-ggaagctcctcctcagcttt-3′ 5′-caggcttccgggtctttaat-3′	198	200
COX5A	5′-gcatgcagacggttaaatga-3′ 5′-agttcctccggagtggagat-3′	152	200
GAPDH	5′-ctttgccaacttccttctgc-3′ 5′-ttgattttggagggatctcg-3′	238	200
CI	5′-cgccacctagcgtctctatc-3′ 5′-tgaaaatccggtcttcatcc-3′	213	200
PGK1	5′-ctgtgggggtatttgaatgg-3′ 5′-cttccaggagctccaaa-3′	198	200
SIRT1	5′-tgtggtagagcttgcattga-3′ 5′-gcctgttgctctcctcatta-3′	153	200

2.14. Statistical analysis

Results were presented as means and SD of three to fifteen independent experiments. For statistical analysis, Origin 2018G (OriginLab, Northampton, USA) was used. Data were checked on normal distribution (Anderson-Darling test) and homogeneity of variance (Levene's test). The data of samples treated with extracts were analyzed for significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$) compared to either the oxidant-treated control (DCF assay) or respective solvent treated control (alamarBlue, intracellular ATP and MMP assay) by one-sample *t*-test (one-sided). In the case of HRR, CS activity and qRT-PCR extract treated samples were compared to the respective solvent control using an unpaired one-sided *t*-test. In case of inhomogeneity of variance Welch's correction was implemented.

3. Results

3.1. Chemical characterization of extracts from winery by-products

Polyphenol-rich extracts were prepared from winery by-products of *Vitis vinifera* L. cv. Riesling (pomace, stems, vine leafs and vine shoots) from a single vineyard. Methanolic solid–liquid extraction coupled to solid phase extraction using XAD16N adsorber resin yielded extracts of 4.4 g/100 g dm of pomace (PE), 4.7 g/100 g dm of stems (SE), 8.2 g/100 g dm of vine leaves (VLE) and 1.2 g/100 g dm of vine shoots (VSE).

Results from analysis of the TPCs of the extracts by the Folin-Ciocalteu method are presented in Fig. 1. All extracts contained noticeable amounts of phenolic compounds, ranging from 432 to 665 mg

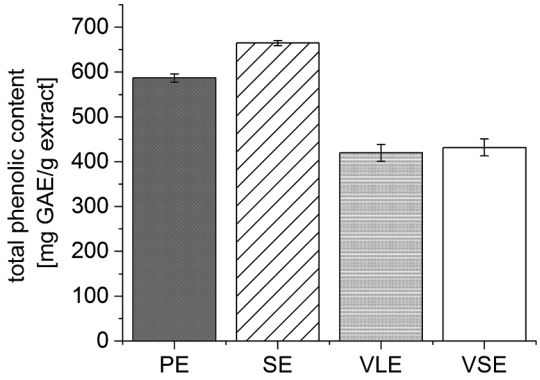

Fig. 1. Total phenolic content (TPC) of extracts from winery by-products expressed as mg gallic acid equivalents (GAE)/g extract (PE: pomace extract; SE: stem extract; VLE: vine leaf extract; VSE: vine shoot extract); $n = 3$ (mean ± SD).

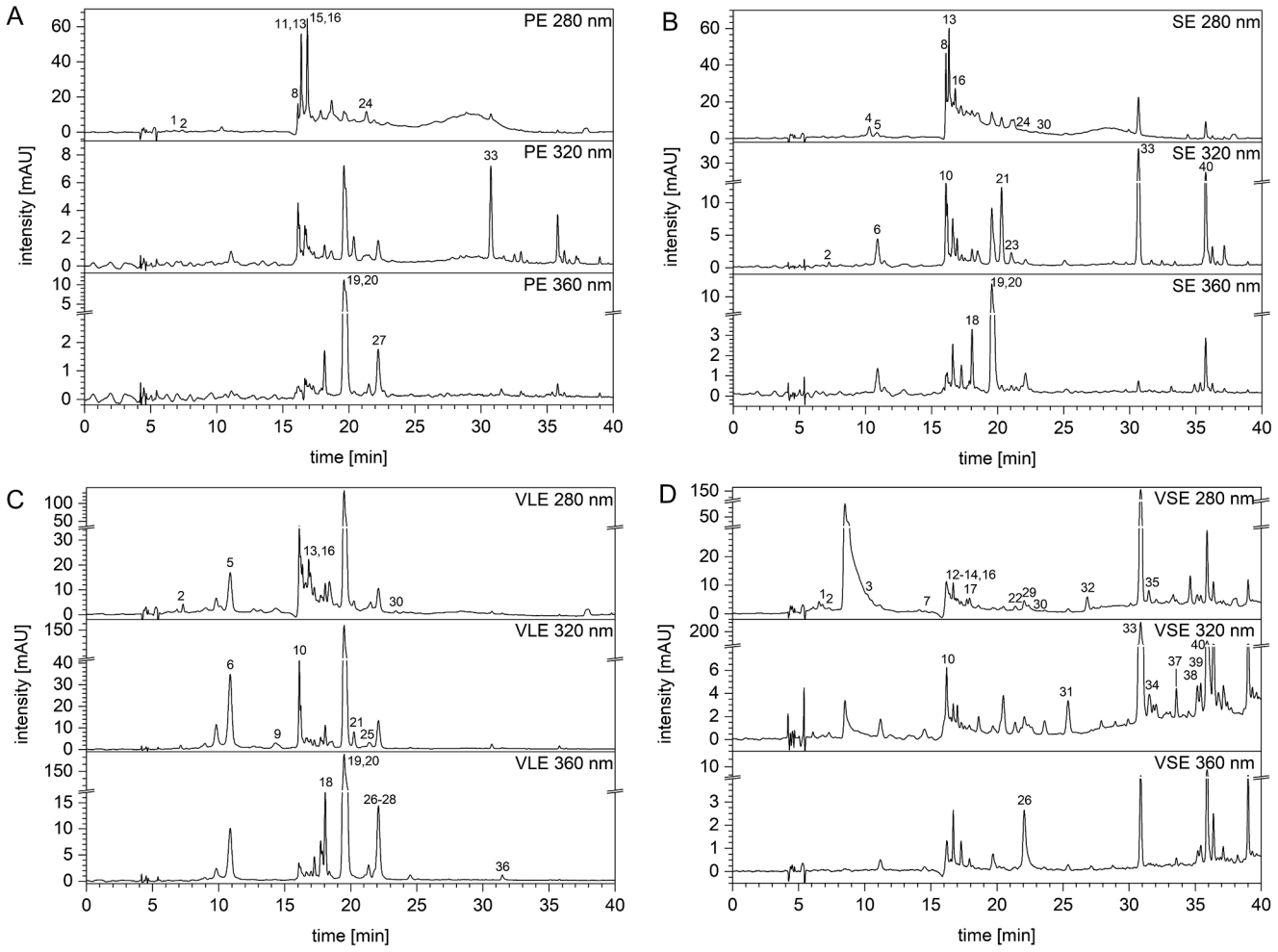

Fig. 2. HPLC-DAD chromatograms (λ = 280, 320, 360 nm) of (A) pomace extract (PE), (B) stem extract (SE), (C) vine leaf extract (VLE), (D) vine shoot extract (VSE).

GAE/g extract (SE > PE > VSE > VLE). Fig. 2 shows HPLC-DAD chromatograms of pomace, stem, vine leaf, and shoot extracts recorded at 280, 320 and 360 nm. Table 2 presents the mass spectral data of compounds identified in the respective extracts by HPLC-ESI-MS/MS in the negative mode. Identification of the polyphenolic compounds was based on comparison of MS and MS/MS data, retention time and UV spectra with commercially available standards and published data. In PE, a total of 12 different phenolic compounds were tentatively identified. Besides the diastereoisomers catechin and epicatechin with $[M-H]^-$ molecular ions at *m/z* 289, three procyanidin B dimers with $[M-H]^-$ at *m/z* 577 and (epi)catechin gallate with molecular ion at *m/z* 441 were identified, as described in the literature (Kammerer, Claus, Carle, & Schieber, 2004; Rockenbach et al., 2012). In addition to benzoic acid derivatives gallic acid ($[M-H]^-$ *m/z* 169) and dihydroxybenzoic acid hexoside ($[M-H]^-$ *m/z* 315), three flavanol derivatives were characterized, including quercetin-*O*-glucuronide ($[M-H]^-$ *m/z* 477), quercetin-*O*-hexoside ($[M-H]^-$ *m/z* 463) and kaempferol-*O*-hexoside ($[M-H]^-$ *m/z* 447). The stilbene *trans*-resveratrol ($[M-H]^-$ *m/z* 227) was also assigned (Kammerer et al., 2004).

Altogether, 17 phenolic compounds were tentatively identified in SE and the polyphenolic profile partly agreed with compounds identified in PE. SE also contained catechin and epicatechin ($[M-HM-H]^-$ *m/z* 289), a procyanidin B dimer ($[M-H]^-$ *m/z* 289), (epi)catechin gallate ($[M-H]^-$ *m/z* 441), dihydroxybenzoic acid hexoside ($[M-H]^-$ *m/z* 315), quercetin-*O*-glucuronide ($[M-H]^-$ *m/z* 477), quercetin-*O*-hexoside ($[M-H]^-$ *m/z* 463) and *trans*-resveratrol ($[M-H]^-$ *m/z* 227). Another identified flavanol derivative was (epi)gallocatechin ($[M-H]^-$ *m/z* 305) (Tomaz & Maslov, 2016). Furthermore, two hydroxybenzoic acid isomers with $[M-H]^-$ at *m/z* 137, rutin ($[M-H]^-$ *m/z* 609) and taxifolin-*O*-rhamnoside ($[M-H]^-$ *m/z* 449) were present (Makris, Boskou, Andrikopoulos, & Kefalas, 2008; Sun, Liang, Bin, Li, & Duan, 2007). $[M-H]^-$ molecular ions at *m/z* 311 and *m/z* 295 were identified as two hydroxycinnamoyltartaric acid derivatives caftaric acid and *trans*-coutaric acid, respectively (Barros, Gironés-Vilaplana, Texeira, Baenas, & Domínguez-Perles, 2015; Ostberg-Potthoff et al., 2019). In addition, a $[M-H]^-$ molecular ion at *m/z* 389 was identified as *trans*-piceid and at *m/z* 453 as ε-viniferin, a resveratrol-resveratrol homodimer, as reported previously in grape stems of different grape varieties (Billet et al., 2018; Makris et al., 2008; Püssa et al., 2006).

In VLE, 17 polyphenolic compounds were tentatively identified, as summarized in Table 2, including hydroxybenzoic acid ($[M-H]^-$ *m/z* 137), dihydroxybenzoic acid hexoside ($[M-H]^-$ *m/z* 315), caftaric acid ($[M-H]^-$ *m/z* 311), coutaric acid ($[M-H]^-$ *m/z* 295), catechin and epicatechin ($[M-H]^-$ *m/z* 289), rutin ($[M-H]^-$ *m/z* 609), quercetin-*O*-glucuronide ($[M-H]^-$ *m/z* 477), quercetin-*O*-hexoside ($[M-H]^-$ *m/z* 463), kaempferol-*O*-hexoside ($[M-H]^-$ *m/z* 447), *trans*-piceid and a second resveratrol-hexoside ($[M-H]^-$ *m/z* 389). Kaempferol-*O*-glucuronide with $[M-H]^-$ *m/z* 461) and quercetin and ellagic acid showing $[M-H]^-$ at *m/z* 301 were also detected (Aouey, Samet, Fetoui, Simmonds, & Bouaziz, 2016; Ostberg-Potthoff et al., 2019). Thus, flavonols were the dominant class of polyphenolic compounds in VLE in the present study, consistent with literature data (Aouey et al., 2016; Kocsis et al., 2015).

A total of 23 phenolic compounds were tentatively identified in VSE. Stilbenes are reported to be a dominant class of polyphenols in

Table 2
Identification of polyphenolic compounds in pomace (PE), stem (SE), vine leaf (VLE) and vine shoot extract (VSE) by HPLC-ESI-MS/MS (negative mode, t_R: retention time, CE: collision energy; for details, see Materials and Methods section).

Peak	Compound (tentative identification)	t_R (min)	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)	CE (eV)	PE	SE	VLE	VSE
1	gallic acid	7.0	169	125, 124	−20	x			x
2	dihydroxybenzoic acid hexoside	7.3	315	151, 153, 152, 108, 109	−25	x	x	x	x
3	dihydroxybenzoic acid	10.3	153	109, 108	−20				x
4	(epi)gallocatechin	10.8	305	125, 179, 137, 219, 164	−20		x		
5	hydroxybenzoic acid	11.2	137	93	−15		x	x	
6	caftaric acid	11.2	311	179, 149	−15		x	x	
7	dihydroxybenzoic acid	14.8	153	109, 108	−15				x
8	procyanidin B dimer	16.2	577	289, 245, 125, 407, 425	−30	x	x		
9	*cis*-coutaric acid	14.4	295	149, 163	−10			x	
10	*trans*-coutaric acid	16.3	295	163	−15		x	x	x
11	procyanidin B dimer	16.5	577	289, 407, 125, 425, 175, 161	−30	x			
12	dihydroxybenzoic acid	16.5	153	109, 108	−15				x
13	catechin	16.6	289	109, 125, 203, 151, 123, 205, 137, 221, 245	−30	x	x	x	x
14	brevifolin carboxylic acid	16.7	291	247	−15				x
			$[M-H-CO_2]^-$						
			247	191, 219, 173, 145, 147	−35				
15	procyanidin B dimer	16.9	577	289, 407, 222, 125, 161, 329, 425	−30	x			
16	epicatechin	17.0	289	109, 125, 203, 123, 137, 151, 245, 205, 221, 149	−30	x	x	x	x
17	*p*-hydroxybenzaldehyde	18.1	121	92	−35				x
18	rutin	18.1	609	300, 301	−35		x	x	
19	quercetin-*O*-glucuronide	19.4	477	301	−20	x	x	x	
20	quercetin-*O*-hexoside	19.6	463	300, 301	−30	x	x	x	
21	*trans*-piceid	20.3	389	227	−20		x	x	
22	hydroxygallic acid	20.9	187	125, 97, 123, 169	−25				x
23	taxifolin-*O*-rhamnoside	21.0	449	151, 285, 303	−20		x		
24	(epi)catechin gallate	21.5	441	169, 289	−20	x	x		
25	resveratrol-hexoside	21.6	389	227	−15			x	
26	ellagic acid	21.8	301	145, 173, 201, 283, 285, 185, 172, 129, 216, 144, 200, 184, 228, 133, 245, 257, 227, 229, 117	−45			x	x
27	kaempferol-*O*-hexoside	22.1	447	285, 284, 255	−30	x		x	
28	kaempferol-*O*-glucuronide	22.2	461	285	−20			x	
29	taxifolin	22.3	303	285, 125, 177	−15				x
30	hydroxybenzoic acid	23.7	137	93	−20		x	x	x
31	piceatannol	25.3	243	159, 201	−35				x
32	resveratrol-piceatannol heterodimer	26.6	469	451, 375, 363	−25				x
33	*trans*-resveratrol	30.9	227	143, 185, 183, 159	−30	x	x		x
34	*cis*-resveratrol	31.5	227	143, 185	−30				x
35	eriodictyol	31.5	287	151, 135	−20				x
36	quercetin	31.5	301	151, 179	−30			x	
37	resveratrol dimer	33.4	453	240, 347, 241, 359, 346, 239, 93, 265, 303	−40				x
38	resveratrol dimer	35.3	453	225, 197, 347, 305, 160, 423, 119, 359, 238, 277	−40				x
39	Naringenin	35.5	271	151, 119	−25				x
40	ε-viniferin	35.9	453	225, 359, 197, 303, 291, 345, 347, 253, 342, 299, 265, 263, 333, 369, 277, 279, 411, 304, 275, 305, 241, 240, 435, 319, 317	−40		x		x

vine shoots (Chaher et al., 2014; Macke, Jerz, Empl, Steinberg, & Winterhalter, 2012; Vergara et al., 2012). Besides *trans*-resveratrol, *cis*-resveratrol ($[M-H]^-$ *m/z* 227) and piceatannol ($[M-H]^-$ *m/z* 243) were identified monomeric stilbenes, as described in the literature (Püssa et al., 2006; Vergara et al., 2012). Furthermore, four compounds were tentatively identified as dimeric stilbene derivatives: a resveratrol-piceatannol heterodimer ($[M-H]^-$ *m/z* 469) and three resveratrol-resveratrol homodimers, including ε-viniferin ($[M-H]^-$ *m/z* 453) (Gorena et al., 2014; Püssa et al., 2006; Vergara et al., 2012). Others were gallic acid ($[M-H]^-$ *m/z* 169), dihydroxybenzoic acid hexoside ($[M-H]^-$ *m/z* 315), hydroxybenzoic acid ($[M-H]^-$ *m/z* 137), *trans*-coutaric acid ($[M-H]^-$ *m/z* 295), catechin and epicatechin ($[M-H]^-$ *m/z* 289), ellagic acid ($[M-H]^-$ *m/z* 301) and *trans*-resveratrol ($[M-H]^-$ *m/z* 227) (Rusjan & Mikulic-Petkovsek, 2015). Besides these compounds, three dihydroxybenzoic acid isomers were identified by $[M-H]^-$ molecular ions at *m/z* 153 (Bonello, Gašić, Tešić, & Attard, 2019). Additionally, brevifolin carboxylic acid ($[M-H]^-$ *m/z* 291), *p*-hydroxybenzaldehyde ($[M-H]^-$ *m/z* 121), hydroxygallic acid ($[M-H]^-$ *m/z* 187), taxifolin ($[M-H]^-$ *m/z* 303), naringenin ($[M-H]^-$ *m/z* 271) and eriodictyol ($[M-H]^-$ *m/z* 287) could be detected.

3.2. Cytotoxicity of the extracts from winery by-products

Effects on the cell viability of HepG2 cells after treatment with winery by-product extracts were determined by the alamarBlue assay and examination of intracellular ATP levels to evaluate non-cytotoxic concentration ranges (cell viability ≥ 80%) for further investigation and show the effects on cellular energy metabolism parameters. The results of cytotoxicity evaluations after 2 h or 24 h extract incubations are presented in Fig. 3. The extracts showed cytotoxic effects in a concentration-dependant manner (PE > SE > VLE > VSE), with decreases in cell viability most pronounced after 24 h incubation. Already at low extract concentrations (2 h ≥ 50 µg/ml; 24 h ≥ 10 µg/ml), decreases in cell viability were observed in the alamarBlue assay. In particular, PE caused distinct cytotoxic effects, resulting in a cell viability below 31% (after 24 h incubation) at 25 µg/ml. The trends observed in cytotoxicity testing using the alamarBlue assay were in line with effects on intracellular ATP levels, as the same range and order of decrease was obtained, i.e., PE > SE > VLE > VSE. Again, PE showed the strongest effects, with an intracellular ATP level of 50% at 30 µg/ml (24 h incubation). Independent of the test system, at both incubation times, VSE produced cell viabilities of < 80% only at high

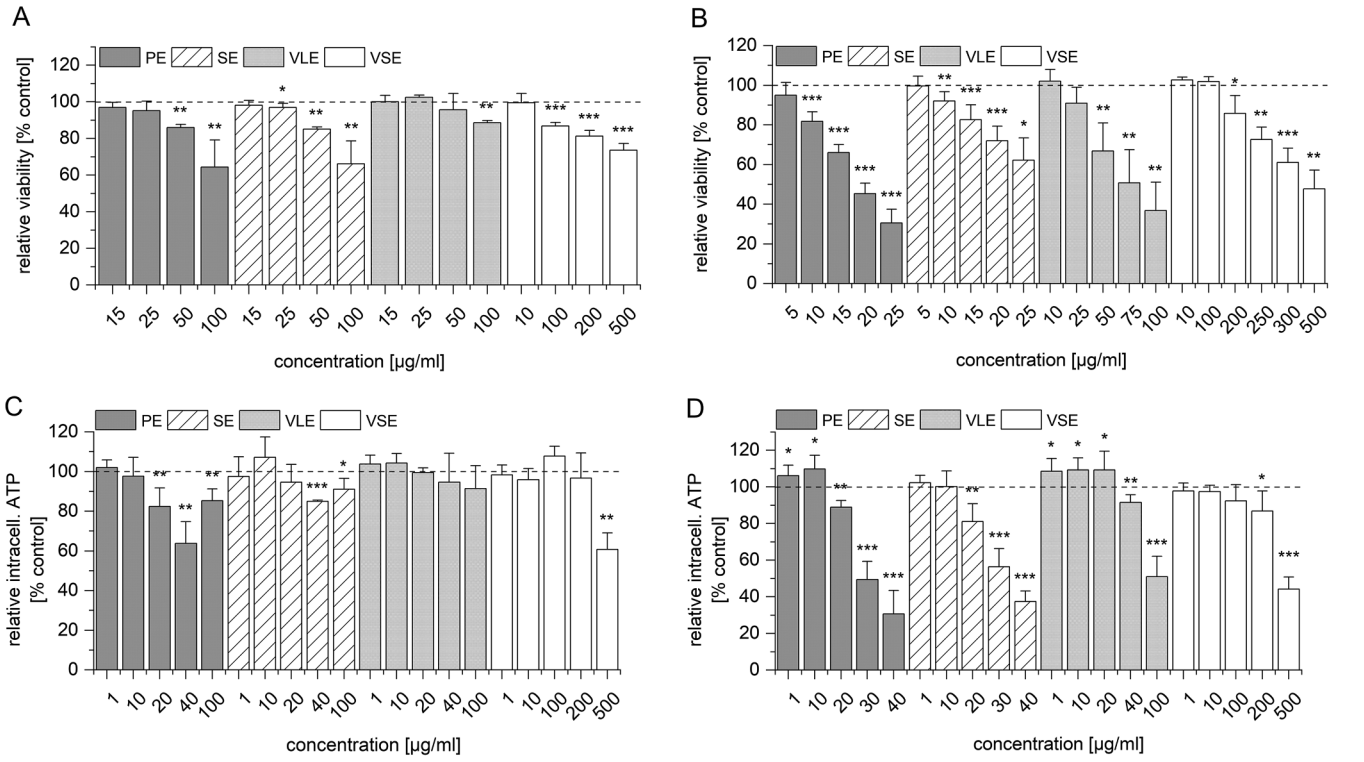

Fig. 3. Assessment of cytotoxicity of extracts from winery by-products in HepG2 cells after (A) 2 h and (B) 24 h incubation by alamarBlue assay. Results are expressed as percent of DMSO treated control; 2 h: n = 3–5; 24 h: n = 3–7 (mean ± SD). Determination of intracellular ATP in HepG2 cells after (C) 2 h and (D) 24 h incubation with pomace, stem, vine leaf and vine shoot extract. Results are expressed as percent of DMSO treated control; 2 h: n = 3–5; 24 h: n = 3–6 (mean ± SD); significantly different from solvent control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (PE: pomace extract; SE: stem extract; VLE: vine leaf extract; VSE: vine shoot extract).

concentrations (> 200 µg/ml).

3.3. Effects on TBH-induced intracellular ROS levels

The effects of treatment with winery by-product extracts on TBH-induced ROS levels are presented in Fig. 4. At both preincubation times, VSE caused significant reductions of TBH-induced ROS levels at concentrations of 100 µg/ml (2 h, $p < 0.001$) and 200 µg/ml (24 h, $p < 0.001$), respectively. For 24 h preincubation with 200 µg/ml VSE, TBH-induced ROS levels were reduced by 41%. In contrast, VLE, PE and SE partially caused significant but not very pronounced reductions of TBH-induced ROS levels. For example, 24 h preincubation with SE effected a significant reduction of TBH-induced ROS levels by 15% at a concentration of 10 µg/ml (24 h, $p < 0.001$). Consequently, VSE showed the highest antioxidative efficacy against TBH-induced intracellular ROS levels. Quercetin (30 µM), used as positive control, reduced intracellular ROS levels by 28% (2 h) and 32% (24 h), respectively.

3.4. Alteration of mitochondrial membrane potential (MMP)

Effects of the extracts on the MMP after 2 h and 24 h incubation are shown in Fig. 5. After 2 h incubation, PE, SE and VLE tended to increase the MMP in a dose-dependent manner, whereas VSE caused a large decline, leading to a reduction to 38% at a concentration of 200 µg/ml. When incubated for 24 h, PE, SE and VLE did not affect the MMP to a great extent. In contrast, 24 h incubation with VSE at concentrations of 100 to 200 µg/ml led to a strong concentration-dependent reduction of

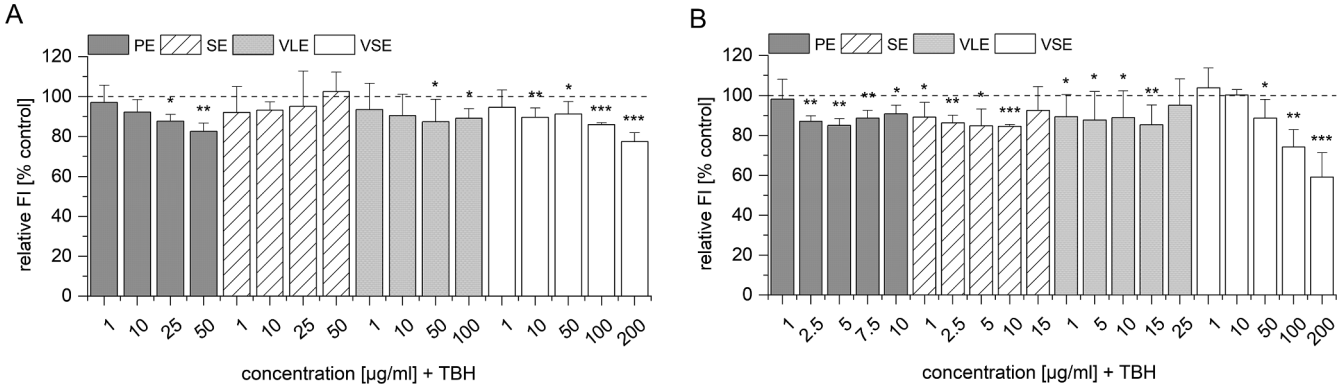

Fig. 4. Modulation of *tert*-butyl hydroperoxide (TBH)-induced intracellular ROS levels in HepG2 cells after (A) 2 h and (B) 24 h preincubation with pomace, stem, vine leaf, and vine shoot extract (PE: pomace extract; SE: stem extract; VLE: vine leaf extract; VSE: vine shoot extract). Results were calculated as percent of TBH treated control; 2 h: n = 3–5; 24 h: n = 3–6 (mean ± SD); significantly lower than TBH-treated solvent control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

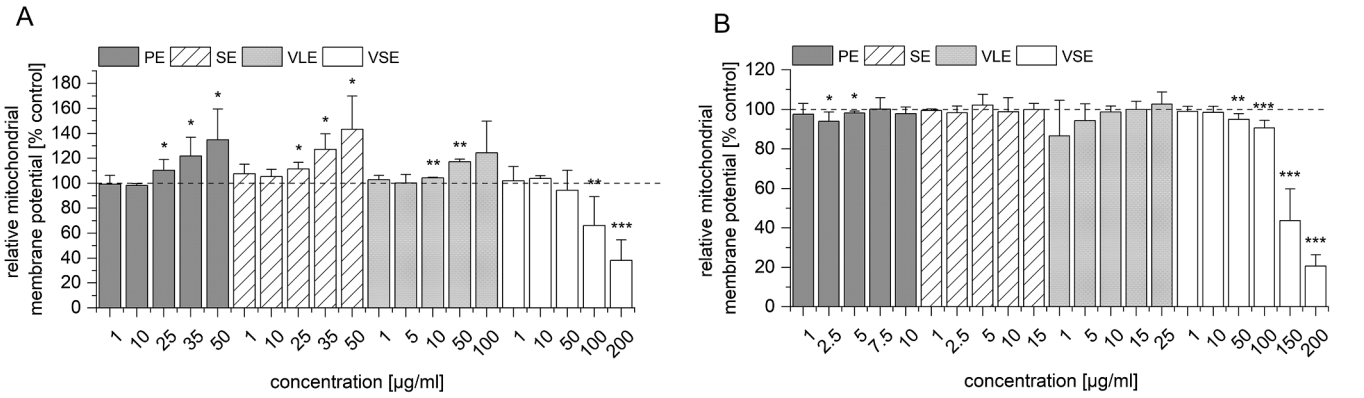

Fig. 5. Modulation of the mitochondrial membrane potential (MMP) in HepG2 cells after (A) 2 h and (B) 24 h incubation with pomace, stem, vine leaf and vine shoot extract (PE: pomace extract; SE: stem extract; VLE: vine leaf extract; VSE: vine shoot extract). Results were calculated as percent of DMSO treated control; 2 h: $n = 3–7$; 24 h: $n = 3–7$ (mean ± SD); significantly different from solvent control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the MMP by up to 79%.

3.5. Effect of vine shoot extract on mitochondrial respiration and citrate synthase activity

The results of HRR are shown in Fig. 6A. From the data normalized to cell-count, 24 h incubation with VSE (250 µg/ml) was found to significantly increase permeabilized, leak (G/M), complex I and complex IV respiration. Overall, cell count normalized mitochondrial function was improved, seen in compensated endogenous and leak respiration and an increased complex IV respiration. CS activity, a quantitative marker of the content of mitochondria, was determined to assess whether an enhanced mitochondrial content was the reason for the examined differences (Williams, Salmons, Newsholme, Kaufman, & Mellor, 1986). CS normalized respiration gives information about the respiration of one single mitochondrion (Park et al., 2014) (Fig. 6A). Endogenous respiration and leak (oligomycin) respiration were significantly but mildly reduced after VSE incubation in comparison to the solvent control ($p < 0.05$). Contrary, increased permeabilized respiration and leak (G/M) respiration has been maintained. CI + CII OXPHOS, ETS and CII ETS respiration decreased numerically but not by statistically significant amounts. VSE impaired both, endogenous and leak respiration in the mitochondrion. Since endogenous respiration represents the physiological respiration of the cell, this finding may explain the observed strong decrease in MMP and ATP levels. Leakage respiration occurs when the inner mitochondrial membrane is damaged or when uncoupling proteins allow proton transfer through the inner mitochondrial membrane (Diano & Horvath, 2012). Thus, uncoupling may further contributed to the low MMP after VSE incubation.

As shown in Fig. 6B, CS activity was significantly ($p < 0.05$) increased in HepG2 cells after 24 h incubation with VSE (250 µg/ml), indicating enhanced mitochondrial mass by induction of mitochondrial biogenesis in VSE treated cells.

The effects of 24 h VSE incubation on the expression of mRNA of CI, CV, CV, and SIRT1 were investigated by qRT-PCR. As shown in Fig. 6C–F, significant changes in mRNA expression levels were detected. VSE treatment caused significant decreases of CI and CV mRNA expression levels ($p < 0.001$), whereas the expression levels of CIV ($p < 0.001$) and SIRT1 ($p < 0.01$) were significantly increased.

In summary, the observed increase in mitochondrial content, as evidenced by an increase in CS activity and upregulation of SIRT1, may represent a physiological regulation that counteracts the low MMP and insufficient ability to produce ATP after VSE incubation.

4. Discussion

Re-utilization of waste or by-products during the processing of polyphenol rich foods offer a rich source for compounds that potentially improve cellular functions at the mitochondrial level. Accordingly, we have previously shown that extracts from stabilized rice bran or olive waste-water improves mitochondrial function *in vitro* (Hagl et al., 2015; Schaffer, Müller, & Eckert, 2010) and in vivo (Hagl et al., 2016; Reutzel et al., 2018). Here we investigated extracts from winery by-products (*Vitis vinifera* L. cv. Riesling) on mitochondrial functions in human hepatocellular carcinoma (HepG2) cells. Winery by-products are rich in polyphenolic compounds. Therefore, assessing the biological activities of phytochemical extracts from winery by-products may help to identify alternative uses for them. Since mitochondria are the main source of intracellular ROS, effects of mitochondria-targeting compounds, such as antioxidants, are of increasing interest. In the present study, we investigated the polyphenolic compositions and biological activities of extracts from winery by-products in HepG2 cells, focusing on the effects on mitochondrial functions. The investigated winery by-products were obtained from pomace, stems, vine leaves and vine shoots of the white grape variety *Vitis vinifera* L. cv. Riesling from a single vineyard. All these plant materials are potent sources of polyphenolic compounds, but the polyphenolic profile and content vary depending on the specific grape variety, geographical location, soil and other environmental factors. In general, TPCs of extracts strongly depend on the extraction process used. In the present study, solid phase extraction was performed to enrich the extracts in polyphenolic compounds and remove interfering substances, resulting in extracts with high TPCs (Fig. 1; PE: 587 mg GAE/g; SE: 665 mg GAE/g; VLE: 420 mg GAE/g; VSE: 432 mg GAE/g). In previous studies, a broad range of TPCs for extracts from winery by-products were reported. For example, PEs showed TPCs ranging from 24 to 618 mg GAE/g extract (Anastasiadi, Chorianopoulos, Nychas, & Haroutounian, 2009; Ayuda-Durán et al., 2019; Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Thimothe, Bonsi, Padilla-Zakour, & Koo, 2007; Xu, Burton, Kim, & Sismour, 2016), SEs from 17 to 584 mg GAE/g extract (Anastasiadi et al., 2009; Apostolou et al., 2013; Goutzourelas et al., 2015; Llobera, 2012; Vázquez-Armenta et al., 2017), VLEs from 61 up to 1205 mg GAE/g extract (Farhadi, Esmaeilzadeh, Hatami, Forough, & Molaie, 2016; Nieto, Jaime, Arranz, Reglero, & Santoyo, 2017; Pari & Suresh, 2008) and VSEs from 53 to 225 mg GAE/g extract (Farhadi et al., 2016; Ju et al., 2016; Zhang, Fang, Wang, Li, & Zhang, 2011). Overall, the TPCs of the extracts in the present study were in the upper range of TPCs reported in the literature.

Regarding the polyphenolic profiles of the extracts (Table 2), most of the identified compounds were previously described as constituents of extracts from winery by-products. Several groups have investigated the chemical composition of pomace (consisting out of grape skins and seeds) from *Vitis vinifera* L. cv. Riesling. For instance, gallic acid, catechin, epicatechin, epicatechin gallate, procyanidin B dimers,

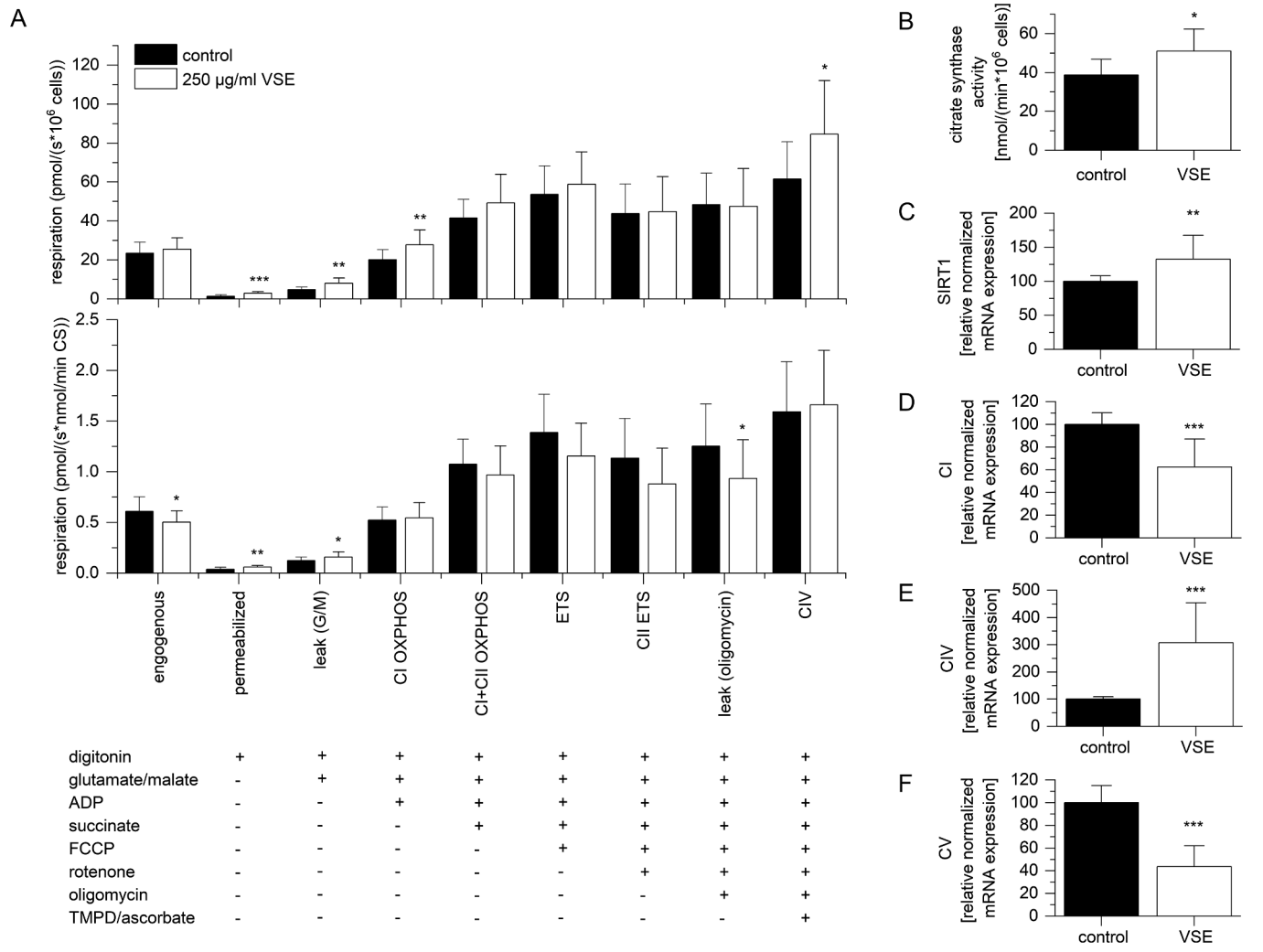

Fig. 6. (A) Respiration of HepG2 cells after 24 h incubation with 250 µg/ml vine shoot extract (VSE) and solvent control (DMSO) measured with an Oxygraph-2 k (Oroboros, Austria) normalized by cell-count and citrate synthase activity. Addition of a substance into the chambers of the Oxygraph is marked with a plus sign. For a detailed protocol, see the Materials and Methods section; n = 10–11 (mean ± SD). (B) Mitochondrial content marker citrate synthase (CS) activity; n = 6 (mean ± SD). (C–F) Relative normalized mRNA expression levels of VSE treated HepG2 cells determined using quantitative real-time PCR in comparison to solvent control cells; n = 13–15 (mean ± SD). G: glutamate; M: malate; CI to CV: complex I to complex V; ETS: electron transport system; SIRT1: Sirtuin 1. Differences were determined by independent one-sided t-test relative to the solvent control: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

quercetin-*O*-glucuronide, quercetin-*O*-hexosides, kaempferol-*O*-hexoside, and *trans*-resveratrol have all been reported (Corrales, Fernandez, Vizoso, & G., Butz P., Franz, Charles M. A. P., Schuele E., , 2010; Garcia-Jares et al., 2015; Kammerer et al., 2004; Wittenauer, Mäckle, Sußmann, Schweiggert-Weisz, & Carle, 2015). Additionally, caftaric acid, protocatechuic acid, quercetin, kaempferol, rutin, isorhamnetin-*O*-hexoside and *trans*-polydatin, amongst others, have been reported in the skins and seeds of the same grape variety (Corrales et al., 2010; Kammerer et al., 2004; Wittenauer et al., 2015) but were not verified in PE in the present study. Flavanols have been described as the dominant class of polyphenols in methanolic PEs, with catechin and epicatechin being the most prominent (Wittenauer et al., 2015). All these findings correlate with our results. A high number of the identified compounds in PE were flavanols, and in the UV-chromatograms of PE, the highest absorption intensity was detected at 280 nm, indicating the presence of flavanols or hydroxybenzoic acids.

The compounds reported for PE were almost all present in SE as well. Additionally, in SE, compounds such as hydroxybenzoic acids, caftaric acid, coutaric acid, (epi)gallocatechin, rutin, resveratrol-hexoside, taxifolin-*O*-rhamnoside, and ε-viniferin were identified. These results are in accordance with literature data (Anastasiadi et al., 2012; Apostolou et al., 2013; Billet et al., 2018; Domínguez-Perles et al., 2016; Goutzourelas et al., 2015; Jara-Palacios, Rodríguez-Pulido, Hernanz, Escudero-Gilete, & Heredia, 2016; Püssa et al., 2006; Souquet, Labarbe, Le Guerneve, Cheynier, & Moutounet, 2000; Spatafora, Barbagallo, Amico, & Tringali, 2013). SEs have been reported to contain considerable amounts of phenolic acids, flavanols, e.g., catechin, epicatechin and procyanidins, flavonols, especially quercetin derivatives, and stilbenes, e.g., *trans*-resveratrol and ε-viniferin (Apostolou et al., 2013; Goutzourelas et al., 2015; Jara-Palacios et al., 2016). The identified polyphenolic compounds of VLE were also in agreement with literature data (Anđelković, Radovanović, Anđelković, & Radovanović, 2015; Balík et al., 2009; Dresch et al., 2018; Katalinic et al., 2012; Katalinić et al., 2009; Kocsis et al., 2015; Schoedl, Forneck, Sulyok, & Schuhmacher, 2011). In leaves, flavonols, especially quercetin- and kaempferol-derivatives, have been found to be the main flavonoids (Kocsis et al., 2015). In previous studies, catechin, epicatechin, ellagic acid, hydroxybenzoic acids, hydroxybenzaldehyde, piceatannol, resveratrol, naringenin and resveratrol dimers were reported as ingredients of VSE, amongst others (Cebrián, Sánchez-Gómez, Salinas, Alonso, & Zalacain, 2017; Delgado-Torre, Ferreiro-Vera, Priego-Capote, Pérez-Juan, & Luque de Castro, 2012; Gabaston et al., 2017; Ju et al.,

2016; Lambert et al., 2013; Luque-Rodriguez et al., 2006; Rusjan & Mikulic-Petkovsek, 2015; Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2014; Zhang et al., 2013). Many of the identified compounds of the VSE were in agreement with data from the literature. However, to the best of our knowledge, brevifolin carboxylic acid and hydroxygallic acid have not previously been identified as constituents in vine shoots. For taxifolin and eriodictyol, the presence of glycosides has been described (Rusjan & Mikulic-Petkovsek, 2015).

Concerning the biological activities of extracts from winery by-products, many studies have been performed, mainly on pomace, followed by stem, leaf and finally vine shoot extracts. In our study, we investigated winery by-products of the white grape variety *Vitis vinifera* L. cv. Riesling owing to its importance in German viticulture and because winery by-products from white varieties are less well studied.

The results of the alamarBlue assay and intracellular ATP level determination (Fig. 3) revealed that all four extracts exhibited dose-dependent cytotoxic effects against HepG2 cells in the order (PE > SE > VLE > VSE) within the tested concentration ranges (1–500 μg/ml). PE and SE, having higher TPCs compared to VLE and VSE, showed distinct cytotoxic potentials after 24 h incubation at a concentration of 25 μg/ml, decreasing the cell viability to 31% and 63%, respectively. Previous studies, mostly performed with intestinal cells, have demonstrated cytotoxic effects of extracts from winery by-products at concentrations of 1.0–320 μg/ml (Empl et al., 2017; Jara-Palacios et al., 2015; Martins et al., 2017; Stagos et al., 2014), although in some studies, no cytotoxic potentials were detected (Chakraborty, Joseph, Naushad, & Abraham, 2016; Slavin, Bourguignon, Jackson, & Orciga, 2016). The wide variation in the cytotoxicity of extracts from winery by-products may be caused by factors such as grape variety, extraction method, polyphenolic profiles, cell line and length of exposure, which can have huge impacts on the obtained results. For instance, a purified white grape pomace extract with TPC of 365 mg/g extract was shown to decrease cell viability of Caco-2 cells after 24 h incubation with 50 μg/ml to 40% (Jara-Palacios et al., 2015). The higher cytotoxicity of the PE in the present study may be attributable to differences in cell type and phenolic compositions, amongst others. Various polyphenols have been shown to exhibit cytotoxic effects against HepG2 cells. For example, resveratrol significantly reduced the cell viability of HepG2 cells after 48 h at 150 μM and higher concentrations (Su et al., 2013). Gallic acid and catechin also showed cytotoxic effects in HepG2 cells after 24 h incubation with 12.5 μM and 100 μM, respectively (Li, Chen, Zhao, & Urmila, 2015; Sun, Zhang, Xie, Zhang, & Zhao, 2016). Under certain conditions, such as high doses, several polyphenols have been shown to exhibit pro-oxidant activities, e.g., as described for quercetin and resveratrol (de Marchi et al., 2009; Martins et al., 2014; Wätjen et al., 2005). However, as quantitative data are not yet available, it is not possible to attribute the cytotoxic effects to individual compounds in the extracts. Thus, the role of pro-oxidative activities and mode of action of polyphenolic compounds in extracts concerning cytotoxicity requires further study.

As mitochondria are the main source of ROS, we focused on antioxidative effects of the polyphenol-rich extracts. Thus, protective effects against TBH-induced ROS levels were investigated. VSE showed pronounced reductions of intracellular ROS at concentrations of 100 μg/ml and higher after 2 and 24 h incubation (Fig. 4). However, the concentration of VSE showing these effects were quite high. Regarding the determined total phenolic content of VSE, a concentration of 100 μg/ml is equal to 43.2 μg GAE/ml. Calculating the corresponding gallic acid concentration as an approximation, this hypothetically is equivalent to 254 μM gallic acid. Thus, among these assumptions the polyphenol concentration showing reductions of TBH-induced ROS levels is out of the range of physiological relevant concentrations. This aspect of physiological relevance of concentrations needs to be considered throughout the entire study as a noticeable limitation. However, it must be noted, that the extracts are complex mixtures, so that concentrations of single compounds are assumed to be lower, and synergistic effects may play a role. The tests were performed in wide concentration ranges, as a general screening was performed. In general, it has to be noted, that the TBH concentration used in this *in vitro* model to induce oxidative stress (250 μM) was quite high as well, so that the obtained results nevertheless are of certain relevance.

Antioxidative effects of extracts from winery by-products have been reported earlier for PE (Martins et al., 2017; Wang et al., 2016) and SE (Domínguez-Perles et al., 2016), grape seed proanthocyanidin extracts (Huang et al., 2012; Shao et al., 2003) and pure compounds, such as quercetin, resveratrol and gallic acid (Alía et al., 2006; Li et al., 2013; Wolfe & Liu, 2007). Pretreatment for 20 h with PE showed concentration-dependent antioxidative effects against TBH-induced intracellular ROS in Caco-2 cells (Wang et al., 2016). In our study, PE, SE and VLE also decreased TBH-induced intracellular ROS levels significantly. There is very little data available regarding the antioxidative effects of VSE *in vitro*. Most previous studies were based on cell-free systems and indicated a high free radical-scavenging and iron chelating activity of VSE (Ju et al., 2016; Sánchez-Gómez et al., 2017). It should be noted that a modified version of the DCF test was performed to investigate the effects on TBH-induced intracellular ROS. Accordingly, no direct assessment of the effects on basal ROS values in the cells and, in particular, in the mitochondria can be made, so that local prooxidative effects cannot be excluded.

Since loss of the MMP represents a key event in the mitochondrial-mediated pathway of apoptosis, the effects of extracts on the MMP of HepG2 cells were assessed after 2 h and 24 h of incubation (Fig. 5). After 2 h, VSE induced a decrease of the MMP at concentrations of 100 μg/ml and higher. In contrast, PE, SE and VLE caused an increase in the MMP after 2 h incubation at concentrations of 25 μg/ml and 10 μg/ml, respectively. After 24 h, VSE modulated the MMP, leading to a distinct decrease at a concentration of 100 μg/ml and higher. Increases of the MMP may be caused by inhibition of ATP synthase (Sánchez-Cenizo et al., 2010). Depolarization of the MMP induced by polyphenolic compounds has been investigated in several studies. Disruption of the MMP has been described for resveratrol, grape proanthocyanidins, grape pomace and grape seed and peel extracts (Espino et al., 2013; Nirmala et al., 2018; León-González, Jara-Palacios, Abbas, Heredia, & Schini-Kerth, 2017; Ma, Tian, Huang, Yan, & Qiao, 2007; Meeran & Katiyar, 2007; Shrotriya et al., 2015; Singh et al., 2011; Yen et al., 2015; Zielińska-Przyjemska, Kaczmarek, Krajka-Kuźniak, Łuczak, & Baer-Dubowska, 2017). For resveratrol, a significant decrease of the MMP was detected at a concentration of 100 μM after 12 h incubation in HepG2 cells and at 30 μM after 24 h incubation in C6 glioma cells (Ma et al., 2007; Zielińska-Przyjemska et al., 2017). Regarding the tested concentrations of the study of Ma et al. (2007) there are limitations with respect to physiological relevance, as investigations with high concentrations of polyphenols may be affected by artifactual effects. VSE disrupts the MMP, which may be the first step in the induction of apoptosis. Since VSE contains resveratrol and stilbenes, the decrease of the MMP may be due to these bioactive compounds.

Investigation of the effects of VSE on the mitochondrial respiratory chain by HRR revealed that cell count normalized respiration was improved after 24 h incubation with 250 μg/ml VSE (Fig. 6). Oxygen consumption of complexes I and IV was significantly increased. Furthermore, respiration in permeabilized cells and the leak state (non-phosphorylating) after the addition of glutamate and malate was significantly ($p < 0.01$) improved. The increase in complex IV respiration is consistent with the significantly elevated CIV mRNA expression levels detected in VSE treated cells. In contrast, although CI activity was increased in the HRR measurements, relative mRNA expression levels of CI were decreased in VSE treated cells, potentially being a defense mechanism against CI-mediated ROS generation. Nevertheless, regarding physiological relevance, the investigated concentration of 250 μg/ml needs to be evaluated critically. Calculating the corresponding gallic acid concentration as an approximation based on total phenolic content, 250 μg/ml VSE hypothetically is equivalent to

635 μM gallic acid. Such relatively high concentrations may be associated with further effects, which not necessarily occur under physiological concentrations.

For better evaluation of the results of HRR, CS activity was additionally determined as a marker of mitochondrial mass. A significant ($p < 0.05$) increase in activity of CS in VSE treated cells was verified. This indicates a higher mitochondrial mass in VSE treated cells compared to control cells, induced by increased mitochondrial biogenesis, as SIRT1 mRNA expression levels were significantly ($p < 0.01$) increased. Several studies have confirmed that resveratrol, the main component of VSE, activates mitochondrial biogenesis through the SIRT/PGC1 pathway (Lagouge et al., 2006). CS normalized respiration of complex I and II as well as uncoupled respiration was numerically, but not statistically, reduced. Endogenous respiration and leak respiration in the presence of oligomycin were significantly reduced. Only the permeabilized and leak (G/M) states were significantly increased after 24 h VSE incubation. Therefore, to compensate reduced respiration chain complex activities VSE seems to induce mitochondrial biogenesis in HepG2 cells, resulting in improved respiration (Fig. 6A). It should be noted that the effects on CS, HRR and the MMP occurred in the same concentration range (200–250 μg/ml VSE). The observed overall increase of respiration might have resulted in reduced vulnerability of complex V against VSE, explaining the higher VSE dose necessary to reduce ATP levels in HepG2 cells (Fig. 3C/D). After 24 h incubation with 250 μg/ml VSE, the mRNA expression level of complex V was significantly ($p < 0.001$) reduced compared to control cells, which correlates with the numerically decreased ATP levels after 24 h incubation with 200 μg/ml VSE.

As the activities of complexes CI–CIV of the mitochondrial respiratory chain represent the driving force for generation of ATP by ATP synthase (complex V), the detected impairment in complex activities may have been caused by a local pro-oxidative effect of substances contained in the extract on mitochondrial structures. In addition, the overall stabilized respiration potentially may have resulted in reduced ROS generation of the respiratory chain. This has to be evaluated in further investigations since we did not examine endogenous ROS formation. However, this effect may have contributed to the reduction of exogenous ROS formation after VSE incubation (see above).

5. Conclusions

To conclude, extracts from winery by-products were prepared and their chemical composition and biological activities regarding mitochondrial function were analyzed. In PE, SE, VLE and VSE, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavanols and stilbenes were identified, amongst others. All four extracts showed concentration-dependent cytotoxic effects, resulting in a reduction of mitochondrial integrity and intracellular ATP levels. All four extracts showed protective effects against TBH-induced oxidative stress, whereby the effects were the most pronounced when preincubating with VSE, which also led to a reduction of the MMP. Moderate interference of mitochondrial functions by VSE was detected using HRR and qRT-PCR and increased CS activity was also determined. These results indicate an increase of mitochondrial mass with concurrent partially negative effect on mitochondrial respiration. However, additional studies are needed to assess the biological activities of extracts from winery by-products, especially concerning mitochondrial functions. In addition, effects of the extracts on fat storing capabilities of cells would be interesting to determine, since the β-oxidation of fatty acids takes place in mitochondria. Quantification of the polyphenolic compounds in the extracts is essential to elucidate the effects of each pure substance and investigate the mechanistic basis of the observed effects. Taken together, the observed antioxidant and mitochondria affecting properties of the winery by-products suggest that they may be interesting sources of bioactive compounds needing further investigation. HepG2 cells were employed in this study as a suitable model revealing high amounts of organelles. Investigating crude extracts from *Vitis vinifera* only limited information is generated due to the facts that complex absorption and/or phase I and II metabolism were not taken into account by our investigations. But first insights were generated and of importance for further studies with e.g. intestinal cells and/or metabolites.

6. Ethics statement

Our research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Christine Fuchs: Investigation, Methodology, Formal analysis, Validation, Writing – original draft. **Tamara Bakuradze:** Funding acquisition, Supervision, Writing – review & editing. **Regina Steinke:** Investigation. **Rekha Grewal:** Investigation, Formal analysis, Writing – review & editing. **Gunter P. Eckert:** Conceptualization, Supervision, Writing – review & editing. **Elke Richling:** Funding acquisition, Project administration, Conceptualization, Supervision, Resources, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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